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PROFESSOR WILLIAM HUGHES (Orcid ID : 0000-0003-0951-9768)

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REVIEW

A mechanistic framework to explain the immunosuppressive effects of neurotoxic pesticides on bees

Tobias Pamminger[1], Christina Botías[1,2], Dave Goulson[1] and William OH Hughes[1*]

[1]*School of Life Sciences, University of Sussex, Brighton. BN1 9QG, UK*

[2] *Estación Biológica de Doñana (EBD-CSIC), 41092 Sevilla, Spain*

* Corresponding author. Email: william.hughes@sussex.ac.uk

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Short title: Influence of neurotoxic pesticides on bee diseases

Abstract

1. There is growing concern that declines in some managed and wild bee pollinator populations threaten biodiversity, the functioning of vital ecological processes and sustainable food production on a global scale.

2. In recent years, there has been increasing evidence that sub-lethal exposure to the neurotoxic class of insecticides (neonicotinoids) can undermine pollinator immunocompetence and amplify the effects of diseases, which have been suspected to be one of the drivers of pollinator declines. However, exactly how neonicotinoids might inhibit pollinator immunity remains elusive.

3. Here we put forward a mechanistic framework to explain the effects of neurotoxic pesticides on insect immunocompetence. We propose that there is a close ontogenetic connection between the cellular arm (haemocytes) of the insect immune and nervous systems, and that this connection makes the immune system of pollinators and other insects inherently susceptible to interference by neurotoxins such as neonicotinoids at sublethal doses.

4. Investigation of this connection is urgently needed to confirm the validity of this framework, and develop a clear, mechanistically-informed understanding of the interplay between neonicotinoids and disease ecology in pollinators. This in turn may enable us to develop strategies to mitigate impacts of neurotoxins on pollinators and/or enhance their impacts on pests.

Key-words: pollinator, immunity, pathogens, neonicotinoids, bee health

Of pollinators, pesticides and parasites

Pollinators play an essential role in the sexual reproduction of most angiosperm species while obtaining pollen and nectar rewards from their flowers (Ollerton, Winfree & Tarrant, 2011). This mutualism is not only crucial for the maintenance of biodiversity, but also provides important ecosystem services, with an estimated 35% of global food production relying on insect pollination (Klein et al., 2007). On the basis of abundance and foraging behaviour, bees are arguably the most effective pollinator group, and threats to managed and wild bees have been more extensively studied than other pollinator taxa (Rader et al., 2016).

Over the last decade, pollinator health has attracted increasing attention following the realization that managed honeybee colony deaths are becoming more frequent in some areas, while some wild pollinator populations are also in decline (Potts et al., 2010; Vanbergen et al., 2013). Numerous global factors are thought to contribute to this alarming phenomenon, including habitat loss and fragmentation, suboptimal diet, diseases, and the detrimental effects of sub-lethal, chronic pesticide exposure (Goulson, Nicholls, Botías & Rotheray, 2015 Vanbergen et al., 2013;). Besides affecting pollinator navigation abilities (Fischer et al., 2014; Henry et al., 2012; Jin et al., 2015; Stanley et al., 2016), cognitive abilities (Decourtye et al., 2004; Gauthier, 2010; Farooqui et al., 2013; Kessler et al., 2015; Piiroinen & Goulson, 2016; Stanley, Smith & Raine, 2015; Williamson, Baker & Wright, 2013; Williamson et al., 2013) and reproduction (Baron, Raine & Brown, 2017; Sandrock et al., 2014; Whitehorn et al., 2012), the immune suppressive effect of sub-lethal pesticide doses and the resulting elevated susceptibility of pollinators to pathogens (Baron, Raine & Brown, 2017; Brandt, Gorenflo, Siede, Meixner & Büchler, 2016; Di Prisco et al., 2013; Doublet et al., 2015; Fauser-Misslin et al., 2014; Hernández López et al., 2017; previous studies summarized in James & Xu, 2012) are of particular concern (Sánchez-Bayo et al., 2016).

In times of a globally operating economy, managed pollinators are shipped around the

world, often carrying their native parasite communities with them (Fürst et al., 2014; McMahon et al., 2015; Wilfert et al., 2016). Commercially managed honey bees carry a wide range of parasites (e.g. *Varroa* mites) and diseases including viral (e.g. deformed wing virus, DWV; Wilfert et al., 2016), fungal (e.g. *Nosema ceranae*; Fürst et al, 2014), bacterial (e.g. *Paenibacillus larvae*; Hernández López et al., 2017) and protozoan (e.g. *Crithidia bombi*; Fausser-Misslin et al., 2014) pathogens, some of which can spread to native bee populations (Fürst et al., 2014; McMahon et al., 2015). Such spillover effects are particularly concerning in intensified western agricultural landscapes. Pollinator immunity in such environments is already likely to be compromised by suboptimal diets caused by monocultural farming practices (Ollerton, Erenler, Edwards, & Crockett, 2014; Scheper et al., 2014), and chronic pesticide exposure (Hladik, Vandever & Smalling, 2016), rendering individuals and entire wild pollinator networks susceptible to pathogen invasion (Di Pasquale et al., 2013; Rundlöf et al., 2015). However, we lack a clear mechanistic understanding of how such general stressors might impair immunity physiologically (Sánchez-Bayo et al., 2016). Illuminating this connection is essential to understand, and ultimately limit, the immunosuppressive effects of such stressors, in particular pesticides (James & Xu, 2012), and thereby help limit the loss of wild and managed pollinator populations. In order to uncover this mechanistic link we have to take a closer look at how insect pollinators generate and regulate their pathogen defence.

How do insect pollinators defend themselves against pathogens?

All animals have evolved highly effective defence mechanisms that protect them against infectious organisms. Invertebrates, including pollinators, are no exception and utilize the innate immune system to fend off invading pathogens (Hoffmann, 1995). This system relies

mostly on genetically encoded factors for pathogen defence and has been traditionally divided into the humoral and cellular defence (Hoffmann, 1995). Humoral defences refer to soluble molecules possessing anti-pathogenic properties, such as anti-microbial peptides (AMPs) and the enzyme cascade regulating melanin generation and haemolymph clotting (PO/PPO pathway) add protection against microparasitic invasions by organisms such as bacteria (Hoffmann, 1995). In addition, insects possess effective means to combat virus infections, with RNAi being the most prominent (Wang et al., 2006). The cellular response includes pathogen detection, digestion (phagocytosis) and encapsulation, which are carried out by specialised blood cells known as haemocytes (Lavine & Strand, 2002; Strand, 2008). These immune cells have a central role in resisting micro- and macroparasite infections (e.g. fungi, protozoa and parasitoids), as well as wound healing (Lavine & Strand, 2002; Strand, 2008). The separation between the defence systems is somewhat artificial as both systems are interlinked and act synergistically to generate a highly effective pathogen defence system (Hoffmann, 1995; Wang et al, 2006).

In recent years, the established view that the innate immune system is simple and nonspecific has radically changed. The documentation of elaborate immune functions such as pathogen group-specific priming that is analogous to immune memory in vertebrates (Kurtz, 2005; Sadd & Schmid-Hempel, 2006), cross-generational (Sadd, Kleinlogel, Schmid-Hempel & Schmid-Hempel, 2005) and temporally fine-tuned (Haine, Moret, Siva-Jothy & Rolff, 2008) immune responses, has generated interest in the organism-wide (systemic) regulation of such complex immune functions (Buchon, Silverman & Cherry, 2014). In vertebrates, complex immune functions are orchestrated by a well-documented neuroendocrinological, innate immune regulatory axis involving a wide range of neurotransmitters and hormones (Sternberg, 2006). There is now evidence for a functionally similar axis in invertebrates utilizing at least two major insect hormones, juvenile hormone (Amdam et al., 2005; Flatt et

al., 2008; Pamminger, Treanor & Hughes, 2016; Rolff & Siva-Jothy, 2002; Tian et al., 2010), and ecdysone (Rus et al., 2013; Sun et al., 2016; Tan, Vlisidou & Wood, 2014), as well as a range of neurotransmitters, including serotonin (Qi et al., 2016), and octopamine (Adamo, 2014; for a comprehensive summary see Adamo, 2012 and Sternberg, 2006). These findings indicate that key functions of the invertebrate innate immune system are under direct neuroendocrinological control (similar to vertebrates), not only enabling the systemic orchestration of immune responses during acute infections, but also likely facilitating immune homoeostasis and appropriate adjustments during internal (nutrient) and external (environmental) stress events (Adamo, 2012, 2014; Buchon, Silverman & Cherry, 2014; Demas, Adamo & French, 2011).

Although most work on insect innate immune regulation has been carried out on a handful of model organisms (including *Drosophila*, *Anopheles* and *Bombyx*), its ancestry suggests that a similar regulatory axis is likely to exist in most or all invertebrate pollinators (Malagoli, Mandrioli, Tascedda & Ottaviani, 2015). Such a regulatory axis, depending on the ability to receive and likely generate neuroendocrinological signals by immunorelevant tissues, provides a potential back-door by which neurotoxic pesticides (often targeting hormone or neurotransmitter receptors) could compromise pollinator immunity directly by interfering with essential immune-regulatory processes. This link has not yet been investigated.

Neurotoxins and disease

Chemical pesticides are intended to effectively suppress populations of pest insects and maintain high crop yields. The most commonly used insecticides today are neurotoxins, which have increased their market share in recent years (Jeschke, Nauen, Schindler & Elbert,

2010). In particular, one class of neurotoxic pesticides, the neonicotinoids, have become a focus of attention from scientists, policy makers and the public (Blaquière et al., 2012; EASAC, 2015; Fryday, Tiede & Stein, 2015; Goulson, 2013). Neonicotinoids target the nicotinic acetylcholine receptors (nAChR) in the invertebrate central nervous system and cause overstimulation at low doses, and receptor blockage, paralysis and death at higher doses (Matsuda et al. 2001). Being systemic (absorbed by the crop plant and then incorporated in multiple plant tissues), these pesticides are present in nectar and pollen of flowering crops, and pollinators foraging on these resources will be chronically exposed to them (Botías et al., 2015; Goulson, 2013; Long & Krupke, 2016; Sánchez-Bayo & Goka, 2014), although the dosage of exposure may often be low (Carreck & Ratnieks, 2014). Although bees have a range of physiological means to deal with pesticide exposure (e.g. detoxification; Cresswell, Robert, Florance & Smirnoff, 2014), the documentation of detrimental effects of sub-lethal neonicotinoid exposure, including immune inhibition, in a range of managed and wild pollinators suggests that this class of potent pesticides is likely to be more harmful to beneficial invertebrates than was previously thought (Goulson, 2013; Goulson, Nicholls, Botías & Rotheray, 2015; Sánchez-Bayo et al., 2016). In particular, the immune suppressive effects of low, field-relevant concentrations of neonicotinoids, have been shown to directly undermine bee resistance to a wide range of diseases caused by fungal (Alaux et al., 2010; Aufauvre et al., 2014; Vidau et al., 2011), protozoan (Fauser-Misslin et al., 2014), bacterial (Hernández López et al., 2017), and viral (Alburaki et al., 2015; Di Prisco et al., 2016; Dively et al., 2015) infections (Table 1; Figure 1). Most of the data available are based on studies of honeybees and bumblebees (Table 1), but the effects likely extend to other pollinators as well given the conserved nature of the invertebrate immune system (Hoffmann, 1995). In the majority of these infections (bacterial, fungal and protozoan) the cellular arm of the invertebrate immune defence (haemocytes) plays a major role in resisting

pathogen infections by means of nodulation and encapsulation (Hoffmann, 1995; Lavine & Strand, 2002), and the central role of haemocytes makes them a likely and, surprisingly, so far overlooked target for neurotoxic interference.

Haemocytes and their connection to neurones

Little is known about the developmental origin of haemocytes (Negri et al., 2016), but they share a close connection with the nervous system (Malagoli, Mandrioli, Tascedda & Ottaviani, 2015). An intimate link between these two systems is known from a wide range of invertebrate systems, with haemocytes being actively involved in neurogenesis and neuroregeneration (Corley & Lavine, 2006; Da Silva et al., 2015). This strong connection between haemocytes and neurones is further supported by findings in the crayfish *Procambarus clarkii*, in which transdifferentiation of circulating haemocytes into functional neurones (without reverting to a pluripotent state) has recently been demonstrated (Benton et al., 2014). These findings are supported by the fact that haemocytes can synthesize neurotransmitters (Qi et al., 2016), possibly including acetylcholine (Pamminger, Basley, Goulson & Hughes, 2017), and express a wide range of neuroendocrinological receptors, making them responsive to both hormonal and neurotransmitter signalling (Flatt et al., 2008; Rolff & Silva-Jothy, 2002; Rus et al., 2013). These signalling pathways have been found to be essential to regulate haemocyte behaviour such as phagocytosis and their migratory behaviour, both during their maturation (differentiation) as well as during acute infections (Adamo, 2012, 2014; Demas, Adamo & French, 2011; Flatt et al., 2008; Malagoli, Mandrioli, Tascedda & Ottaviani, 2015; Hoffmann, 1970; Qi et al., 2016; Stefano, Cadet & Scharrer, 1989; Stefano et al., 1989). There is support from a wide range of invertebrate and vertebrate animals for a functional neuro-haemocyte regulatory connection (Malagoli, Mandrioli,

Tascedda & Ottaviani, 2015), suggesting that a functionally similar relationship likely exists in all pollinator insects.

This close connection between the two types of functionally distinct tissue presents a possible explanation for why haemocytes might be particularly susceptible to the neurotoxic effects of pesticides. Haemocytes express a wide range of hormone and neurotransmitter receptors, including the subunits of nAChR receptor (Pamminger, Basley, Goulson & Hughes, 2017; Shi et al., 2012; Xu et al., 2016). Consequently, it seems likely that haemocytes possess functional nAChR receptors. Neonicotinoids show high affinity for nAChR receptors, and haemocytes have indeed been shown to respond to neonicotinoids (Brandt, Gorenflo, Siede, Meixner & Büchler, 2016). Independent of the actual exposure route (oral or contact), these pesticides have to cross the haemolymph in order to reach their intended target in the insect's central nervous system. On their way they will inevitably encounter haemocytes circulating in the haemolymph. If we are correct that haemocytes have functional nAChR receptors or other receptors to which neurotoxic pesticides have affinity, then when they encounter haemocytes they will bind to, activate and consequently interfere with haemocyte functionality. Such off-target effects during critical stages in haemocyte development and migration, and during acute infections, make this logical mechanistic connection a prime candidate for causing a wide range of the observed immunosuppressive effects in pollinators.

Impacts beyond acute infections

Maturation of the innate immune system

In order to mount an effective immune response at the adult stage, the insect immune system has to go through a complex maturation process including haemocyte migration, proliferation and differentiation. Work in *Drosophila melanogaster* shows that embryonic haemocytes

spawn from mesodermal tissue during only three discrete time windows in the course of development (Krzemień et al., 2007; Makhijani & Brückner, 2012; Traver & Zon, 2002). These embryonic haemocytes, guided by the peripheral nervous system, migrate to populate the haemopoietic pockets (Gold & Brückner, 2014; Makhijani et al. 2011). These cell populations persist during metamorphosis and build the basis for the majority of the differentiated adult haemocytes (Ghosh et al., 2015; Parsons & Foley, 2016). Given that many vital immune functions of insects likely rely on larval-derived haemocyte populations for pathogen protection (Evans & Wood, 2011), and that the capability to generate haemocytes during the pupal and adult stage is likely limited, a disruption during these critical developmental windows will have long-lasting and potentially irreversible effects on the immunocompetence of the adult (Figure 1).

Metamorphosis

In addition to their central function in the innate immune system, haemocytes are vital during a second critical developmental process in invertebrates: metamorphosis (Truman & Riddiford, 1999). All holometabolous insects, after a varying number of juvenile moults, pupate and transition into their adult form (Ghosh et al., 2015). During this process, the internal morphology is completely restructured, involving a series of apoptotic events. Haemocytes perform vital roles during this stage, being involved in the functional restructuring itself (Kurata, Saito & Natori, 1992), and in cleaning up the remnants of these apoptotic cells (Sonnenfeld & Jacobs, 1995). Their failure to do so could result in a build-up of decaying cellular material in the pupae (Sonnenfeld & Jacobs, 1995). Consequently, disruption of haemocyte proliferation, maturation and migration by pesticides during early developmental stages might not only directly impair immune functions, but also have delayed long-term effects during metamorphosis (Figure 1).

Future directions

We are only beginning to understand how the complex interactions of biotic and abiotic stressors influence pollinator health (Goulson, Nicholls, Botías & Rotheray, 2015). This problem needs to be approached at multiple levels utilizing a cross-disciplinary approach. On the molecular level, we need confirmation of the expression of functional nACh receptors in different tissue types (Pamminger, Basley, Goulson & Hughes, 2017), which might offer an explanation for why neonicotinoids affect such a wide range of fitness-relevant functions in pollinator. This should focus on the presence of functional receptors at the cell surface of non-neural tissues and the demonstration that pesticides and their metabolites can activate such receptors at field-realistic exposure levels. On the physiological level, it needs to be investigated if such a potential activation results in impairment of haemocyte performance during immune challenges (e.g. motility and encapsulation rate). On the organismal level, we need to understand if such potential pesticide interference results in increased mortality during infection processes, ideally testing a range of different relevant pathogens including virus, bacterial and fungal infections. Moving beyond the individual, neurotoxic insecticides have been shown to alter social interactions between worker bees (Ingram et al., 2015), which has the potential to affect the transmission dynamics of pathogens within bee colonies. Furthermore, for social bees, we need to understand how differential exposure of the different castes translates into impacts on colony fitness. Ultimately we need to understand how such effects scale up to the ecological level; how the combined exposure to pesticides and pathogens affect plant-animal mutualistic networks (Gegear, Otterstatter & Thomson, 2006; Stanley & Raine, 2016). We do not at present know whether neonicotinoid exposure of entire networks of pollinators (for example the broad suite of insects that might visit a

neonicotinoid-treated oilseed rape crop) impacts upon disease epidemiology both within and between pollinator species, something we might predict since many pollinators share pathogens and interspecific transmission may occur via shared flower use (Graystock, Goulson & Hughes, 2015).

Over the past years, a great deal of research effort has been directed towards understanding the interaction between multiple factors simultaneously acting on adult insect pollinators (Goulson, Nicholls, Botías & Rotheray, 2015), but little is known about the effect of pesticides on the developing larva (Peng & Yang, 2016). All of the developmental effects outlined above could have implications for juvenile bees because they feed as larvae on contaminated pollen and nectar (Zhu et al., 2014). However, the majority of studies have only focussed on the short-term toxic effects of pesticides and their immediate effects during infection on adults in a handful of model systems (Baron, Raine & Brown, 2014; Fauser-Misslin et al., 2014; Vidau et al., 2011). These study designs and system choice will not be able to detect delayed effects during larval development and later in adult life, making an adequate assessment of these effects an important target for future studies especially in non-model species. The limited data available indicate that pesticides can interfere with key insect neurological functions and have long-lasting effects beyond the developmental stage that is exposed (Gregorc & Ellis, 2011; Tomé et al., 2012; Yang et al., 2012). Recently, the first evidence for long-lasting effects of developmental exposure to neonicotinoids has emerged (Doublet et al., 2015). Given that the developmental environment can have a profound impact on immunocompetence, both during further development (Lee, Simpson & Wilson, 2008) and in the adult insect (Fellous & Lazzaro, 2010), it is logical to expect that pesticide exposure during the development of larvae will have detrimental effects in their adult life, but further research is needed to demonstrate this. In honeybees, pesticide residues in stored food may not be fed to larvae until months after collection, potentially resulting in impaired

immune function in the resulting adults and disease epizootics occurring months or even years after the pesticide was initially used on a crop, obscuring the causative link between the two.

While extended exposure is expected to have detrimental effects, it is likely that susceptibility to pesticides varies over time (Rondeau et al., 2014). It is well known that the neonicotinoid class of insecticides exhibit pronounced variation in toxicity depending on the moulting stage (Grewal, Power & Shetlar, 2001), possibly reflecting the variation in importance of the acetylcholine receptors during these periods. Recent research has provided strong evidence for the immediate effects of neonicotinoid exposure on larval immunocompetence (Hernández López et al., 2017). It should be of high priority to investigate delayed effects, as early deleterious effects on developing innate immunity may not only affect larval development but also compromise adult immunocompetence. Such potential effects highlight the importance of designing experiments that encompass multiple developmental stages to understand the potentially delayed costs of sub lethal pesticide exposure on pollinator health.

Assessing the impact of pesticides on pollinator health by sub-lethal chronic exposure requires a holistic view of the problem. In recent years, research has started to uncover the side-effects of sub-lethal neonicotinoid exposure on cognitive abilities, including pollinator navigation and learning (Decourtye et al., 2004; Farooqui et al., 2013; Fischer et al., 2014; Gauthier, 2010; Goulson, Nicholls, Botías & Rotheray, 2015; Henry et al., 2012; Jin et al., 2015; Kessler et al., 2015; Piiroinen & Goulson, 2016; Stanley, Russell, Morrison, Rogers & Raine, 2016; Stanley, Smith & Raine, 2015; Williamson, Baker & Wright, 2013; Williamson et al., 2013). A similar intensity of research effort now needs to be focused on the sub-lethal effects of neurotoxic pesticides on insect immunity. The mechanistic framework that we have proposed here, with the innate immune system being in some ways a functional extension of

the nervous system, provides an explanation for why such effects may occur and highlights where further research is needed to gain a more in-depth understanding of the pesticide–immunity–pathogen interaction. Although most evidence for this proposed framework comes from neonicotinoids, it is likely that other neurotoxic insecticides, and possibly even the acaricides routinely used in apiculture, may have similar effects. Such effects need to be tracked beyond the individual to the group and community levels. There is clearly potential for immunosuppressive effects of neurotoxic pesticides to have profound impacts on abundance of a broad suite of beneficial pollinating insects, with consequent ecological impacts from the resulting effects on seed set of wild plants. Further research is urgently needed to appropriately assess the danger of sub-lethal pesticide exposure to both managed and wild pollinators, in order to maintain the health of their populations, the ecosystem services that they provide, and sustainable crop production to meet the food demands of a growing world population.

Authors' contributions

T.P. developed the idea and wrote the first draft of the manuscripts. C.B., D.G. and W.O.H.H. contributed ideas and to revisions of the manuscript.

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Data accessibility

This MS has no data associated with it.

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Figure legends:

Figure 1: Summary of the potential effects of neurotoxic pesticides on pollinator immunity. Based on results from older developmental stages (late larvae to adult), we expect the early developmental stages to suffer from increased pathogen susceptibility (Table1), resulting from reduced haemocyte performance during acute infections (spreading number, composition, spreading behaviour, pathogen detection, encapsulation and nodulation). In addition, we expect compromised haemocyte functionality during development (e g. mobility and migration), resulting in complications during the formation of the haemopoetic organs and metamorphosis. The same mechanisms may compromise transgenerational immune priming as well. These effects are likely to be additive, resulting in increasing risk of mortality before reaching adulthood (red arrows). We have summarized the documented and predicted effects for each developmental stage (Table 1). All pictures used are public domain and distributed under a CC0 licence.

Table 1:

Table 1: Evidence for immunosuppressive effects of neonicotinoid pesticides in pollinators

Pesticide	Host species	Stage affected	Haemocytes affected	Pathogen	Study
Clothianidin	Honey bee (*Apis mellifera*)	Adult	Yes	Virus (Deformed Wing Virus)	Di Prisco et al., 2013
Imidacloprid	Honey bee (*Apis mellifera*)	Adult	Unkown	Virus (Deformed Wing Virus)	Dively et al., 2015
Imidacloprid	Honey bee (*Apis mellifera*)	Adult	Unknown	Fungi (*Nosema ceranae*)	Alaux et al., 2010
Imidacloprid	Honey bee (*Apis mellifera*)	Adult	Unknown	Fungi (*Nosema ceranae*)	Aufauvre et al., 2014
Imidacloprid	Honey bee (*Apis mellifera*)	Adult	Unknown	Fungi (*Nosema ceranae*)	Pettis et al., 2012
Thiacloprid	Honey bee (*Apis mellifera*)	Adult	Unknown	Fungi (Nosema ceranae)	Vidau et al., 2011
Thiamethoxan	Bumblebee (*Bombus terrestris*)	Adult	Unknown	Protozoa (*Crithidia bombi*)	Fauser-Misslin et al., 2014
Clothianidin	Bumblebee (*Bombus terrestris*)	Adult	Unknown	Protozoa (*Crithidia bombi*)	Fauser-Misslin et al., 2014
Clothianidin	Honey bee (*Apis mellifera*)	Larvae	Yes	Bacteria (*Paenibacillus larvae*)	Hernández López et al., 2017